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Patents ADP number *(if you know it)* 8331985001

If the applicant is a corporate body, give the country/state of its incorporation: United Kingdom

4. Title of the invention

CYTOCHROME C PROTEIN AND ASSAY

5. Name of your agent *(if you have one)*

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Cytochrome C Protein and Assay

Technical Field

The present invention relates to a cytochrome C-reporter fusion protein construct which targets the mitochondria and has a reduced ability to induce apoptosis in a living cell. The fusion construct of the invention can be used in assays for detecting early events in apoptosis in living cells.

Background to the Invention

'Programmed cell death' or apoptosis is a key event in multicellular organisms, defining a genetically encoded cell death program which is morphologically, biochemically and molecularly distinct from necrosis (Vermes et al., J Immunol Meth., (2000) 243, 167-190). The characteristic morphological signs of apoptosis (cellular shrinkage, membrane blebbing, nuclear condensation and fragmentation) are the results of a complex biochemical cascade of events which is an integral part of physiological homeostasis.

Apoptosis ensures an equilibrium between cell proliferation and cell death, thus playing a regulatory role in the control of the size of cell populations and tissues. Aberrations in cell death signalling, in membrane or cytoplasmic receptors, or alterations in genes that govern apoptosis are involved in the pathogenesis of congenital malformations and many acquired diseases (Haanen & Vermes, Eur J Obstetr.Gynecol., (1996) 64, 129-133). Too little apoptosis may result in malignancies (Tomlinson & Bodmer, Proc. Natl. Acad.Sci. USA, (1995) 92, 11130- 11134), Leukemias (Sachs, Proc. Natl. Acad. Sci. USA, (1996) 93, 4742- 4749) or the resistance to anticancer therapy (Pahor et al., Lancet, (1996) 348, 493-497). Too much apoptosis can result in immune deficiency (Meyaard et al., Science (1992) 257, 217-219) and degenerative conditions (Griffith et al., Science, (1995) 270, 1189-1192).

There is therefore considerable interest within the medical, pharmaceutical and toxicological sciences in developing a greater understanding of the events which trigger and regulate apoptosis. Furthermore, there is a need to develop new techniques which can be used to identify, quantify and characterise agents which can modulate this phenomenon.

Assays for Detecting Apoptosis

A large number of assays have been developed to detect the onset of programmed cell death (Sgone & Wick, Int Arch Allergy Immunol., (1994) 105, 327-332; Sgone & Gruber, Exp Gerontol., (1998) 33, 525-533). These assays are based upon a wide range of events associated with cell death and have traditionally included light and electron microscopy with vital staining and nuclear dyes. Biochemical methods are often employed, for example based upon DNA laddering or degradation, DNA end labelling techniques (e.g. TUNEL –terminal deoxynucleotide transferase dUTP Nick End labelling), nuclease activity and lactate dehydrogenase enzyme release.

Flow cytometry tends to be the most widely used method (Vermes et al., J Immunol Methods, (2000) 243, 167-190) for detecting and quantifying apoptosis because it is amenable to screening large numbers of cells. This fluorescence - based technique employs vital dyes, antibodies to apoptotic enzymes (e.g. caspases) and single stranded DNA breaks, together with probes for measuring calcium flux and phospholipid redistribution.

Flow cytometry allows *in vivo* analysis of cells in suspension, one at a time, at rates of 1000 to 10,000 cells/s. However, one problem with flow cytometry is that it can only be used in cells in suspension, such as liquid cell cultures and cells derived from the hemopoietic system. Furthermore flow cytometry of tissue cells requires physical and enzymatic manipulation to get the cells in suspension, which by itself may trigger apoptosis, necessitating checks by conventional light or fluorescence microscopy.

Existing methods are thus based upon late events in apoptosis (e.g. DNA degradation, caspase assays) and many require cellular fixation and staining with specific

antibodies/dyes. None of the techniques described above provide a homogeneous living cell assay, based upon the early events in apoptosis, in real time nor discriminate from cellular necrosis. Furthermore, none of these assays are amenable to high throughput live cell screening which is required to test large numbers of compounds for their ability to modulate apoptosis.

Cytochrome C Translocation as a Marker for Apoptosis

Cytochrome C is a nuclear encoded protein which is targeted to the mitochondria where it performs its biological function as an electron carrier. The translocation of cytochrome C from the mitochondria to the cytoplasm in response to apoptotic stimuli is an early and critical step in the commitment of the cell to undergo apoptosis (Li et al., Cell (1997) 91, 479-489). Cytochrome C binds strongly to apoptosis protease activation factor -1 (Apaf-1) in the cytosol (Zou et al., Cell (1997) 90, 405-413). In the presence of cofactors the resulting cytochrome C: Apaf-1 assembles into a multimeric 'apoptosome' that binds and activates a protease zymogen, procaspase-9 (Srinivasula et al., Mol. Cell (1998) 1, 949-957). This results in the activation of the 'caspase cascade' whereby many intracellular substrates are cleaved disabling important cellular processes and breaking down structural components of the cell (Slee et al., J Cell.Biol. (1999) 144, 281-292; Skulachev, FEBS Lett., (1998) 423, 275-280). A schematic diagram illustrating the mitochondrial role in apoptosis is shown in Figure 1.

Recent *in vitro* studies (Kluck et al., J. Biol. Chem., (2000), 275, 16127-16133; Yu et al., J.Biol.Chem., (2001), 276, 1304-13038) have identified the molecular determinants involved in the cytochrome C: Apaf-1 interaction. Horse cytochrome C has been shown to be highly homologous to human cytochrome C and can initiate caspase activation, whereas yeast cytochrome C did not measurably bind to Apaf-1 nor activate caspase (Yu et al., J Biol Chem., (2001) 276, 13034-13038). These studies focussed on the key differences between horse and yeast cytochrome C and used site directed mutagenesis to generate mutant variants which were subsequently analysed with respect to their ability to activate caspase. The results from this *in vitro* work indicated that residue 7, 25, 39, 62-65 and 72 were critical amino acids for cytochrome C:Apaf-1 interaction. Notably the mutation K72A showed no detectable binding or caspase-9 activation. While the mutation of lysine

72 to alanine abolished the interaction between cytochrome C and Apaf1, respiratory function of cytochrome C was unaffected.

Although the authors demonstrated reduced binding to Apaf-1 and caspase-9 activation *in vitro* it is not known whether the cytochrome C mutant proteins would behave in a similar manner in living cells, targeting the mitochondria and not inducing apoptosis.

Recent reports by Abdullaev et al. (Biochem J. (2002) 362, 749-754), again based on *in vitro* experiments, indicate that the horse K72 mutants described by Yu et al. (J Biol Chem., (2001) 276, 13034-13038) show the same level of caspase activation as the wild-type protein if present at 2-12 fold higher concentrations than the wild type protein. In contrast, the authors reported that a horse K4E cytochrome C mutant was inactive in activating caspase and conclude that this mutant, rather than K72 mutants, would be a good candidate for *in vivo* knock-in studies on the role of cytochrome C in apoptosis.

Fluorescent Proteins

The use of Green Fluorescent Protein (GFP) derived from *Aequorea victoria* is now well known for research into many cellular and molecular-biological processes. Cytochrome C-GFP fusions have been used in studies on apoptosis. Thus Heiskanen et al. (J Biol Chem., (1999) 274, 5654-5658) expressed a cytochrome C-GFP fusion, based upon rat cytochrome c, in rat pheochromocytoma-6 (PC6) cells. Induction of apoptosis by staurosporine led to release of the fusion from the mitochondria which was accompanied by mitochondrial depolarisation.

Goldstein et al., (Nat Cell Bio., (2000) 2, 156-160) demonstrated mitochondrial localisation of a cytochrome C–GFP fusion, based upon human cytochrome C, over-expressed in HeLa cells. A range of apoptotic inducers were shown to cause rapid release of cytochrome C-GFP.

Problem to be Addressed

There is a need to develop sensitive assays which are amenable to high throughput screening in living cells and which allow detection and analysis in real time of the early events in apoptosis.

While over-expression of cytochrome C in cells has been demonstrated, the resulting high levels of protein lead to apoptosis and cell death, necessitating the use of complex inducible/transient systems for studies involving up-regulation of cytochrome C levels (Chandra et al., J Biol Chem. (2002) 277, 50842-50854). To date, it has not been possible to generate stable cell lines over-expressing this protein which could be reliably used for screening purposes.

There is thus a need for a cytochrome C protein which, when coupled to a reporter group to form a fusion construct, can be expressed in cells to provide stable cell lines. The resulting construct can act as a biosensor within the cells for early events in the induction or repression of apoptosis. Stable cell lines overexpressing such constructs are suitable for high throughput screening purposes to identify agents which modulate apoptosis.

The present invention addresses this problem and provides a fusion construct which has no observable toxicity to cells and acts as a biosensor to permit the detection of early events in apoptosis in living cells. The stably transformed cells of the invention can still undergo apoptosis due to the presence of endogenous cytochrome C, thus allowing detection and determination of a second apoptotic signalling event whether up or downstream of the cytochrome C translocation.

Summary of the Invention

In a first aspect of the present invention there is provided a cytochrome C-reporter fusion protein construct comprising a modified cytochrome C protein or any functional analogue thereof derived from wild type cytochrome C, wherein the modified cytochrome C targets the mitochondria and has a reduced ability to induce apoptosis in a living cell.

A reportor is to be understood to be any group that is detectable due to its radioactive, fluorescent or luminescent properties or is localisable by a detectable moiety such as a labelled antibody or specific binding compound.

Preferably, the modified cytochrome C binds apotosis protease activation factor-1 (Apaf-1) at least 10 times less than wild type cytochrome C. More preferably, the modified cytochrome C binds Apaf-1 at least 100 times less than wild type cytochrome C. Most preferably, the modified cytochrome C binds Apaf-1 at least 1000 times less than wild type cytochrome C.

Suitably, at least one of the amino acids of the modified cytochrome C at positions 4, 7, 8, 25, 39, 62, 63, 64, 65 and 72 has been mutated relative to the wild type cytochrome C.

Suitably, the modified cytochrome C has an amino substitution or substitutions selected from the group consisting of K4E, K72A, K72L, K72R, K72G, K72X, E62N, K7E-K8E, K25P-K39H, K7A-E62N-K25P, K7A-E62N-K39H, K7E-K8E-E62N, K7A-K25P-E62N, K7A-E62N-K25P-K39H, E62N-T63N-L64M-M65S, K7E-K8E-E62N-K25P-K39H, K7E-K8E-K25P-E62N-T63N-L64M-M65S, K7E-K8E-K39H-E62N-T63N-L64M-M65S and K7E-K8E-K25P-K39H-E62N-T63N-L64M-M65S.

Preferably, the modified cytochrome C comprises the amino acid substitution selected from the group consisting of K7E-K8E-E62N-K25P-K39H, K7E-K8E-K25P-E62N-T63N-L64M-M65S, K7E-K8E-K39H-E62N-T63N-L64M-M65S and K7E-K8E-K25P-K39H-E62N-T63N-L64M-M65S.

More preferably,the modified cytochrome C comprises the amino acid substitution selected from the group consisting of K72A, K72L, K72R, K72G and K72X, wherein X represents trimethylation. Most preferably, the modified cytochrome C comprises the amino acid substitution K72A or K72L.

Preferably, modified cytochrome C comprises the amino acid substitution K4E.

Suitably, the reporter is a fluorescent protein or a functional analogue thereof.

It will be understood by the person skilled in the art that a functional analogue of a fluorescent protein will include, but is not limited to, any detectable fluorescent protein fragment formed in a protein fragment complementation assay as described, for example, in US 6,270,964, US 6,428,951 and US 6,294,330.

Preferably, the fluorescent protein of the present invention is a Green Flourescent Protein (GFP) derived from *Aequoria Victoria*, *Renilla reniformis* or other members of the class *Anthozoa* (Labas et al., Proc.Natl.Acad.Sci, (2002), 99, 4256-4261).

US 6172188 describes variant GFPs wherein the amino acid in position 1 preceding the chromophore has been mutated to provide an increase in fluorescence intensity. These mutants result in a substantial increase in the intensity of fluorescence of GFP without shifting the excitation and emission maxima. F64L-GFP has been shown to yield an approximate 6-fold increase in fluorescence at 37 ^{o}C due to shorter chromophore maturation time.

One mutant, commonly termed Enhanced Green Fluorescent Protein (EGFP), contains the mutations F64L and S65T (Cormack, B.P. et al., Gene, (1996), 173, 33-38). EGFP has been optimised for expression in mammalian systems, having been constructed with preferred mammalian codons.

Suitably, the fluorescent protein is selected from the group consisting of Green Fluorescent Protein (GFP), Yellow Fluorescent Protein (YFP), Blue Fluorescent Protein (BFP), Cyan Fluorescent Protein (CFP), Red Fluorescent Protein (RFP), Enhanced Green Fluorescent

Protein (EGFP) and Emerald. Preferably, the fluorescent protein is either EGFP or Emerald.

GB 2374868 describes GFP derivatives having a triple mutation at F64, S65/E222 and S175 which exhibit enhanced fluorescence relative to wild type GFP when expressed in non-homologous cells at temperatures above 30°C and when excited at about 490 nm. Mutant GFPs produced using the method of the invention provide a means for detecting GFP reporters in mammalian cells at lower levels of expression and/or increased sensitivity relative to wild type GFP.

Preferably, the GFP of the present invention comprises

i) an amino acid substitution at position F64L;
ii) an amino acid substitution at position S175G; and
iii) an amino acid substitution at position E222G.

In a preferred embodiment the fusion construct is either SEQ ID NO: 4 or SEQ ID NO: 6.

In one embodiment, the reporter is localisable by a detectable luminescent, fluorescent or radio-active moiety. Thus, for example, the reporter comprises an immunogenic motif and the detectable moiety may be a luminescent, fluorescent or radio-actively labelled antibody.

Suitably, the reporter comprises a FLAG™, HA, HIS, c-Myc, VSV-G, V5 or a HSV (Sigma-Aldrich) epitope which is localisable by specific labelled antibodies.

In another embodiment, the reporter comprises a cysteine-rich motif and the detectable moiety comprises a labelled biarsenical compound as described by Griffin et al., Science (1998), 281, 269-272) and in US 6,054,271, US 6,008,378 and US5,932,474.

In a second aspect of the present invention, there is provided a nucleotide sequence encoding a protein fusion construct as hereinbefore described.

Preferably the nucleotide sequence is SEQ ID NO: 3 or SEQ ID NO: 5.

In a third aspect of the present invention, there is provided a nucleic acid construct comprises a suitable control region and the nucleotide sequence as hereinbefore described, the sequence being under the control of the control region.

Suitably, the nucleic acid construct is under the control of a promoter selected from the group consisting of native cytochrome C promoter, mammalian constitutive promoter, mammalian regulatory promoter, human ubiquitin C promoter, viral promoter, SV40 promoter, CMV promoter, yeast promoter, filamentous fungal promoter and bacterial promoter.

Preferably, the promoter is the CMV or the SV40 promoter. More preferably, the promoter is the human ubiquitin C promoter.

In a fourth aspect of the present invention, there is provided a replicable vector comprising a nucleic acid construct as hereinbefore described.

Suitably, the vector is a plasmid vector as described by Makrides (Prot Expression & Purif. (1999) 17, 183-202).

Preferably the vector is a viral vector. Suitable viral vectors for use in the invention are described, for example, by Ng et al., Hum Gene Ther. (2000) 11, 693-699 and include cytomegalovirus, Herpes simplex virus, Epstein-Barr virus, Simian virus 40, Bovine papillomavirus, Adeno-associated virus, Adenovirus, Vaccina virus and Baculovirus vector.

In a fifth aspect of the present invention, there is provided a host cell stably transformed with a nucleic acid construct as hereinbefore described.

In a sixth aspect of the present invention, there is provided a host cell transiently transformed with a nucleic acid construct as hereinbefore described.

Suitably, the host cell is selected from the group consisting of plant, insect, nematode, bird, fish and mammalian cell. Preferably the cell is a human cell. More preferably the human cell is selected from the group consisting of Hek, Hela, U2OS and MCF-7. Most preferably the cell is Hek cell line 293 (Hek293).

Suitably, the host cell is capable of expressing the fusion protein as hereinbefore described.

In a seventh aspect of the present invention, there is provided a method for detecting apoptosis in a living cell comprising the steps of

i) culturing a cell transformed to over-express a fusion construct as hereinbefore described;

ii) determining the localisation of the fusion construct within the cell with time;

wherein a change in localisation of the fusion construct within the cell is indicative of apoptosis.

In an eighth aspect of the present invention, there is provided a method for measuring the effect an agent has upon modulating apoptosis in a living cell comprising the steps of

i) culturing a cell transformed to over-express a fusion construct as hereinbefore described;

ii) determining the localisation of the construct within the cell;

iii) treating the cell with the agent and determining the localisation of the construct within the cell;

wherein any difference in the localisation of the construct within the cell relative to control cells untreated with the agent is indicative of the effect the agent has upon modulating apoptosis.

In a ninth aspect of the present invention, there is provided a method for measuring the effect an agent has upon modulating apoptosis in a living cell comprising the steps of

i) culturing a first cell and a second cell which both over-express a fusion construct as hereinbefore described;

ii) treating the first cell with the agent and determining the localisation of the construct within the first cell;

iii) determining the localisation of the construct within the second cell which has not been treated with the agent;

wherein any difference in the localisation of the construct within the first cell and second cell is indicative of the effect the agent has upon modulating apoptosis.

In a tenth aspect of the present invention, there is provided a method for measuring the effect an agent has upon modulating apoptosis in a living cell comprising the steps of

i) culturing a cell transformed to over-express a fusion construct as hereinbefore described;

ii) treating the cell with the agent and determining the localisation of the construct within the cell;

iii) comparing the localisation of the construct in the presence of the agent with a known value for the localisation of the construct in the absence of the agent;

wherein any difference in the localisation of the construct within the cell in the presence of the agent and the known value in the absence of the agent is indicative of the effect the agent has upon modulating apoptosis.

Suitably, the known value according is stored on a database, such as an electronic or optical database.

Suitably, the localisation of the protein fusion is measured by its luminescence, fluorescence or radioactive properties.

The method of the invention is suitable for screening purposes to identify agents which induce or inhibit apoptosis.

In another embodiment of the method of the invention, the localisation of the fusion construct is determined on non-living, fixed cells. Thus, the living cells over-expressing the fusion construct are fixed at specific time points by conventional means and the location of the fusion protein detected using a detectable moiety, such as a labelled antibody or specific binding chemical. In this way, the effect an agent has upon apoptosis can be

determined by comparing localisation of the fusion construct in the presence and the absence of the agent. Alternatively, the effect that the agent has upon apoptosis can be determined by comparing localisation of the construct in the presence of the agent against a known value (for example, one stored on a database) for localisation in the absence of the agent.

Suitably, the agent is a chemical, physical or biological agent. Examples of chemical agents include inorganic and organic compounds, such as drugs, toxins, peptides, proteins and nucleic acids. Physical agents include electromagnetic radiation such as electrical, magnetic and light (UV, gamma, IR, visible) energy. Examples of typical biological agents include viruses, prions, bacteria and fungi which could infect a living cell and modulate apoptosis.

Brief Description of the Drawings

Figure 1. Schematic representation depicting the mitochondrial role in apoptosis.

Figure 2 a) InCell Analyzer 3000 images (ca. 40x magnification) showing cytochrome C (K72A-GFP mutant) labelled mitochondria in Hek 293 cells of clone 2B6.

Figure 2 b) Confocal microscopic image (ca. 60x) cytochrome C-GFP expression in HeLa cells (from Goldstein et al., (Nat Cell Bio., (2000) 2, 156-160))

Figure 3 a)-c). InCell Analyzer 3000 images showing colocalisation of cytochrome C (K72A mutant)-GFP and Tetramethylrhodamine ethyl ester (TMRE) using dual excitation of cytochrome C and TMRE in Hek 293 cells.

Figure 3 a) Cytochrome C (K72A)-GFP

Figure 3 b) TMRE labelled mitochondria

Figure 3 c) overlay of Fig 3a) & 3b) following dual excitation

Figure 4. Nucleic acid sequence encoding wild type cytochrome C (SEQ ID NO: 1)

Figure 5. Amino acid sequence of wild type cytochrome C (SEQ ID NO: 2)

Figure 6. Nucleic acid sequence encoding F64L-S175G-E222G-triGFP–cytochrome C (K72A) construct (SEQ ID NO: 3)

Figure 7. Amino acid sequence of F64L-S175G-E222G-triGFP–cytochrome C (K72A) construct (SEQ ID NO: 4)

Figure 8. Nucleic acid sequence encoding cytochrome C (K72A)- F64L-S175G-E222G-triGFP (SEQ ID NO: 5)

Figure 9. Amino acid sequence of cytochrome C (K72A)- F64L-S175G-E222G-triGFP (SEQ ID NO: 6)

Figure 10. Nucleic acid sequence encoding F64L-S175G-E222G-wild type cytochrome C construct (SEQ ID NO: 7)

Figure 11. Amino acid sequence of F64L-S175G-E222G wild type cytochrome C construct (SEQ ID NO: 8)

Figure 12. Nucleic acid sequence encoding wild type cytochrome C-F64L-S175G-E222G tri GFP (SEQ ID NO: 9)

Figure 13. Amino acid sequence of wild type cytochrome C-F64L-S175G-E222G tri GFP (SEQ ID NO: 10)

Specific Examples

Example 1

Amplification of the cytochrome C gene, fusion to GFP (F64L-S175G-E222G) and introduction of the K72A (APAF-1 binding) mutation.

The fluorescent-cytochrome C mutant fusion proteins of the current invention were produced by joining, in frame, a sequence of the nucleic acid that encodes for the cytochrome C protein to a sequence of the nucleic acid that encodes for a fluorescent protein and then introducing the K72A (APAF- 1 binding) mutation (Kluck et al., J Biol Chem., (2000) 275, 16127-16133)) to the nucleic acid of the fusion construct. A preferred sequence of the human cytochrome C gene is described by Zang and Gerstein (Gene, (2003) 312, 61-72); NCBI Accession number NM_018947. (SEQ ID NO: 1) the encoded protein is shown in SEQ ID NO: 2. Alternative human cytochrome C sequences may be used. In addition, alternative sequences around the start and stop codons of the gene may be used to provide useful restriction enzyme sites for protein fusion. Where such alterations change the amino acid numbering relative to the reference sequence such numbering should be inferred by amino acid alignment with the reference sequence. Preferred sequences of the gene encoding the fluorescent protein include those derived from *Aequorea victoria* published by Chalfie *et al*, (Science, (1994) 263, 802-5), the GFP-F64L-S175G-E222G mutant (GB Patent 2374868), Emerald (Aurora biosciences), EGFP and related mutants (BD Clontech, Palo Alto, CA), and fluorescent proteins from species of *Anthazoa*, for review see Labas *et al*, (PNAS, (2002) 99, 4256-4261).

The cytochrome C gene was amplified by RT-PCR from a mixed human cDNA library using primers CYCS1 and CYCS2 or CYCS1 and CYCS3 according to recognised protocols (Sambrook, J. et al (2001) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press).

CYCS1; 5′-gttgaattcgaccatgggtgatgttgagaaaggc
CYCS2; 5′-gttgttgtcgaccttactcattagtagctttttgag
CYCS3; 5′-gttgttgtcgaccctcattagtagctttttgag

Primer CYCS1 exhibits homology to the 5′ region of the cytochrome C gene and contains both a partial Kozak sequence (Kozak, Cell (1986), 44, 283) and an *Eco*R1 restriction enzyme site. Primer CYCS2 exhibits homology to the 3′ region of the cytochrome C gene and contains a stop codon and *Sal*I restriction enzyme site. Primer CYCS3 exhibits homology to the 3′ region of the cytochrome C gene and contains a *Sal*I restriction enzyme site. The CYCS1-CYCS2 and CYCS1-CYCS3 RT-PCR products were cloned into the corresponding *Eco*RI and *Sal*I sites of the GFP-fusion vectors pCORON1000-GFP-C1 and N1, respectively (Amersham Biosciences, Cardiff, UK) and verified by automated sequencing. These vectors contain a CMV promoter to effect the expression of the GFP fusion and an SV40 promoter to elicit expression of a neomycin resistance marker. The GFP within these vectors is red-shifted and contains the mutations F64L-S175G-E222G as described in GB 2374868.

Cytochrome C (K72A) mutants were generated with the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, Ca, USA) using primers CYCS4 and CYCS5.

CYCS4; 5′-ggagtatttggagaatcccgccaagtacatccctggaacaa
CYCS5; 5′-ttgttccagggatgtacttggcgggattctccaaatactcc

After sequence verification the pCORON1000-GFP-wild type cytochrome C and K72A mutant fusion constructs were sub-cloned into the vector pCORON2100 using the restriction enzymes *Nhe*I and *Not*I. pCORON2100 contains a CMV promoter and an IRES element to drive bicistronic expression of the GFP-fusion protein and a neomycin resistance marker.

The nucleic acid and amino acid sequences of the GFP-cytochrome C constructs obtained are shown in Figures 6 to 13 (SEQ ID NOS: 3-10)

Example 2

Influence of cytochrome C-K72A (APAF-1 binding) mutation upon GFP-fusion protein stable cell line generation in mammalian cells.

Plasmid DNA to be used for transfection was prepared for all constructs using the HiSpeed plasmid purification kit (Qiagen, Westberg, NL). In addition to the constructs in example 1, pCORON1000-GFP and pCORON2100-GFP were used as selection controls. DNA was diluted to 100 ng. μl^{-1} in 18-Megohm water (Sigma, Dorset, UK) and 1 μg used for transfections. For 50-80% confluency on the day of transfection, Hek293 cells were plated at a density of $5x10^4$/well in 6-well plates and incubated overnight. A 1:3 (1 μg : 3 μl) ratio of DNA to FuGene6 reagent (Roche Diagnostics, Basel, Switzerland) was used for each transient transfection reaction; 3 μl FuGene6 was added to 87 μl serum-free DMEM medium (Sigma) (containing penicillin/streptomycin, L-glutamine [Invitrogen, Carlsbad, CA]) and gently tapped to mix, then 10 μl (1 μg) construct DNA was added and again gently mixed. The FuGene6 : DNA complex was incubated at room temperature for 40 minutes and added dropwise, with gentle mixing, directly to the cells without changing the medium. The plates were then gently swirled for even distribution. Cells were monitored for expression after 24 and 48 hours using a Nikon Eclipse TE200 epifluorescent microscope (Nikon, Melville, NY). Cells were passed into 15cm diameter plates and after 24 hours placed under selection with geneticin (G418, 250 ng. μl^{-1}; Sigma). The concentration of geneticin was increased incrementally to 500 ng. μl^{-1} over the following 5-7 days. Selection continued for around 10 days or until cells in the mock-transfected control plates had died. Cloning rings were then used to isolate surviving colonies and cells were expanded through 96-well, 24-well and 6-well plates. Where appropriate second and third rounds of clonal selection were applied.

After the first round of clonal selection surviving cells were visible for transfections containing pCORON1000-GFP, pCORON2100-GFP and the pCORON2100-GFP-N and -C cytochrome C (K72A) mutant plasmids. No surviving colonies were obtained from cells transfected with other plasmid contructs.

Results

Expression studies of this mutated cDNA were performed in pCORON2100 (Amersham Biosciences) in order to utilise the IRES element and facilitate the generation of stable cell lines. A "mixed population" stably expressing cell line under selection with geneticin G418 was continuously cultured for 3 weeks. 15 single clonal cell lines were then isolated.

Hek293 cells containing the pCORON2100-GFP-cytochrome C (K72A) mutants were shown to exhibit stable expression during continuous culture over a 4 month period. The mitochondrial localisation of the reporter fusion proteins was confirmed during this period by co-localisation with the known mitochondrial marker mitotracker red (Molecular Probes, Eugene, OR) using a Microsystems LSM (Zeiss, Thornwood, NY) and a high throughput laser scanning confocal microscope (INCell Analyzer 3000, Amersham Biosciences).

The expression results from one of these stable clones (2B6), using the InCell Analyzer 3000 (Amersham Biosciences, UK) laser scanning confocal imaging system, are shown below in Figure 2. As can be seen from Figure 2a the localisation pattern of expressed cytochrome C-GFP closely resembles that observed by Goldstein et al., (Nat Cell Bio., (2000) 2, 156-160), using a confocal microscope, reproduced in Figure 2b. As expected, the fusion protein demonstrates nuclear exclusion and localises to mitochondria, the cytochrome C-GFP displaying a punctuate pattern of fluorescence (Figure 2a).

Figures 3a and 3b show localisation of the fusion protein construct (a) and the mitochondrial stain TMRE (b) at a concentration of 40nM in the mitochondria of Hek 293 cells. Dual excitation of the cytochrome C-GFP and TMRE in the InCell Analyzer demonstrates co-localisation (Figure 3c).

Claims

1. A cytochrome C-reporter fusion protein construct comprising a modified cytochrome C protein or any functional analogue thereof derived from wild type cytochrome C, wherein said modified cytochrome C targets the mitochondria and has a reduced ability to induce apoptosis in a living cell.

2. The fusion construct of claim 1, wherein the modified cytochrome C binds apoptosis protease activation factor-1 (Apaf-1) at least ten times less than wild type cytochrome C.

3. The fusion construct of claim 1 or 2, wherein the modified cytochrome C binds apoptosis protease activation factor-1 (Apaf-1) at least 100 times less than wild type cytochrome C.

4. The fusion construct of any preceding claim, wherein the modified cytochrome C binds apoptosis protease activation factor-1 (Apaf-1) at least 1000 times less than wild type cytochrome C.

5. The fusion construct of any preceding claim, wherein at least one of the amino acids of the modified cytochrome C at positions 4, 7, 8, 25, 39, 62, 63, 64, 65 and 72 has been mutated relative to the wild type cytochrome C.

6. The fusion construct of claim 5, wherein the modified cytochrome C has an amino substitution or substitutions selected from the group consisting of K4E, K72A, K72L, K72R, K72G, K72X, E62N, K7E-K8E, K25P-K39H, K7A-E62N-K25P, K7A-E62N-K39H, K7E-K8E-E62N, K7A-K25P-E62N, K7A-E62N-K25P-K39H, E62N-T63N-L64M-M65S, K7E-K8E-E62N-K25P-K39H, K7E-K8E-K25P-E62N-T63N-L64M-M65S, K7E-K8E-K39H-E62N-T63N-L64M-M65S and K7E-K8E-K25P-K39H-E62N-T63N-L64M-M65S.

7. The fusion construct of claim 6, wherein the modified cytochrome C comprises the amino acid substitution selected from the group consisting of K7E-K8E-E62N-K25P-K39H, K7E-K8E-K25P-E62N-T63N-L64M-M65S, K7E-K8E-K39H-E62N-T63N-L64M-M65S and K7E-K8E-K25P-K39H-E62N-T63N-L64M-M65S.

8. The fusion construct of claim 6, wherein the modified cytochrome C comprises the amino acid substitution selected from the group consisting of K72A, K72L, K72R, K72G and K72X, wherein X represents trimethylation.

9. The fusion construct of either of claims 6 or 8, wherein the modified cytochrome C comprises the amino acid substitution K72A or K72L.

10. The fusion construct of claim 6, wherein the modified cytochrome C comprises the amino acid substitution K4E.

11. The fusion construct of any preceding claim, wherein the reporter is a fluorescent protein or a functional analogue thereof.

12. The fusion construct of claim 11, wherein said fluorescent protein is selected from the group consisting of Green Fluorescent Protein (GFP), Yellow Fluorescent Protein (YFP), Blue Fluorescent Protein (BFP), Cyan Fluorescent Protein (CFP), Red Fluorescent Protein (RFP), Enhanced Green Fluorescent Protein (EGFP) and Emerald.

13. The fusion construct of either of claims 11 or 12, wherein the fluorescent protein is Enhanced Green Fluorescent Protein or Emerald.

14. The fusion construct of claim 11, wherein the GFP comprises

i) an amino acid substitution at position F64L;

ii) an amino acid substitution at position S175G; and

iii) an amino acid substitution at position E222G.

15. The fusion construct of any preceding claim selected from the group consisting of SEQ ID NO: 4 and SEQ ID NO: 6.

16. The fusion construct according to any of claims 1 to 10, wherein the reporter is localisable by a detectable luminescent, fluorescent or radio-active moiety.

17. The fusion construct according to claim 16, wherein the reporter comprises an immunogenic motif.

18. The fusion construct according to claim 15 or 16, wherein the reporter comprises a cysteine-rich motif.

19. The fusion construct according to claim 16, wherein said detectable moiety comprises a bi- arsenical compound.

20. The fusion construct according to claim 16, wherein said moiety comprises an antibody.

21. A nucleotide sequence encoding a fusion construct of any of the preceding claims.

22. A nucleotide sequence encoding a fusion construct according to claim 15 selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO 5.

23. A nucleic acid construct comprising a suitable control region and a nucleotide sequence according to claim 21 or 22, said sequence being under the control of said control region.

24. A nucleic acid construct according to claim 23 being under the control of a promoter selected from the group consisting of native cytochrome C promoter, mammalian constitutive promoter, mammalian regulatory promoter, human ubiquitin C promoter, viral promoter, SV40 promoter, CMV promoter, yeast promoter, filamentous fungal promoter and bacterial promoter.

25. A nucleic acid construct according to claim 24, wherein said viral promoter is the CMV or the SV40 promoter.

26. A nucleic acid construct according to claim 24, wherein the promoter is the human ubiquitin C promoter.

27. A replicable vector comprising a nucleic acid construct according to any of claims 23 to 26.

28. The replicable vector of claim 27, wherein said vector is a plasmid vector.

29. The replicable vector of claim 27, wherein the vector is a viral vector.

30. The replicable vector of claim 29, wherein said viral vector is selected from the group consisting of cytomegalovirus, Herpes simplex virus, Epstein-Barr virus, Simian virus 40, Bovine papillomavirus, Adeno-associated virus, Adenovirus, Vaccina virus and Baculovirus vector.

31. A host cell stably transformed with a nucleic acid construct according to any of claims 23 to 26.

32. A host cell transiently transformed with a nucleic acid construct according to any of claims 23 to 26.

33. The host cell of claims 31 or 32 selected from the group consisting of plant, insect, nematode, bird, fish and mammalian cell.

34. The host cell of claim 33, wherein said mammalian cell is a human cell.

35. The host cell of claim 34, wherein said human cell is selected from the group consisting of Hek, HeLa, U2OS and MCF-7.

36. The host cell of claim 35, wherein said Hek cell is Hek293.

37. The host cell according to any of claims 31 to 36 capable of expressing the fusion protein of any of claims 1 to 20.

38. A method for detecting apoptosis in a living cell comprising the steps of

i) culturing a cell transformed to over-express a fusion construct according to any of claims 1 to 20;

ii) determining the localisation of the fusion construct within the cell with time;

wherein a change in localisation of the fusion construct within the cell is indicative of apoptosis.

39. A method for measuring the effect that an agent has upon modulating apoptosis in a living cell comprising the steps of

i) culturing a cell transformed to over-express a fusion construct according to any of claims 1 to 20;

ii) determining the localisation of said construct within the cell;

iii) treating the cell with said agent and determining the localisation of the construct within the cell;

wherein any difference in the localisation of the construct within the cell relative to control cells untreated with the agent is indicative of the effect that the agent has upon modulating apoptosis.

40. A method for measuring the effect an agent has upon modulating apoptosis in a living cell comprising the steps of

i) culturing a first cell and a second cell which both over-express a fusion construct according to any of claims 1 to 20;

ii) treating said first cell with said agent and determining the localisation of said construct within the first cell;

iii) determining the localisation of the construct within said second cell which has not been treated with the agent;

wherein any difference in the localisation of the construct within the first cell and second cell is indicative of the effect that the agent has upon modulating apoptosis.

41. A method for measuring the effect an agent has upon modulating apoptosis in a living cell comprising the steps of

i) culturing a cell transformed to over-express a fusion construct according to any of claims 1 to 20;

ii) treating said cell with said agent and determining the localisation of the construct within the cell;

iii) comparing the localisation of the construct in the presence of the agent with a known value for the localisation of the construct in the absence of the agent;

wherein any difference in the localisation of the construct within the cell in the presence of the agent and said known value in the absence of the agent is indicative of the effect that the agent has upon modulating apoptosis.

42. The method according to claim 41, wherein the known value is stored on a database.

43. The method according to any of claims 38 to 42, wherein the localisation of said fusion construct is measured by its luminescence, fluorescence or radioactive properties.

44. The method according to any of claims 39 to 43, wherein said agent induces apoptosis.

45. The method according to any of claims 39 to 43, wherein the agent inhibits apoptosis.

46. The method according to any of claims 38 to 45, wherein the localisation of the protein fusion is determined following fixation of the cells.

47. The method according to any of claims 38 to 46, where the agent is a chemical, physical or biological agent.

Abstract

The present invention relates to a a cytochrome C-reporter fusion protein construct comprising a modified cytochrome C protein which targets the mitochondria and has a reduced ability to induce apoptosis in a living cell. The invention also relates to nucleic acid constructs encoding such protein fusions and cells stably transfected with such constructs. The stably transfected cells of the invention can be used in assays to detect apoptosis.

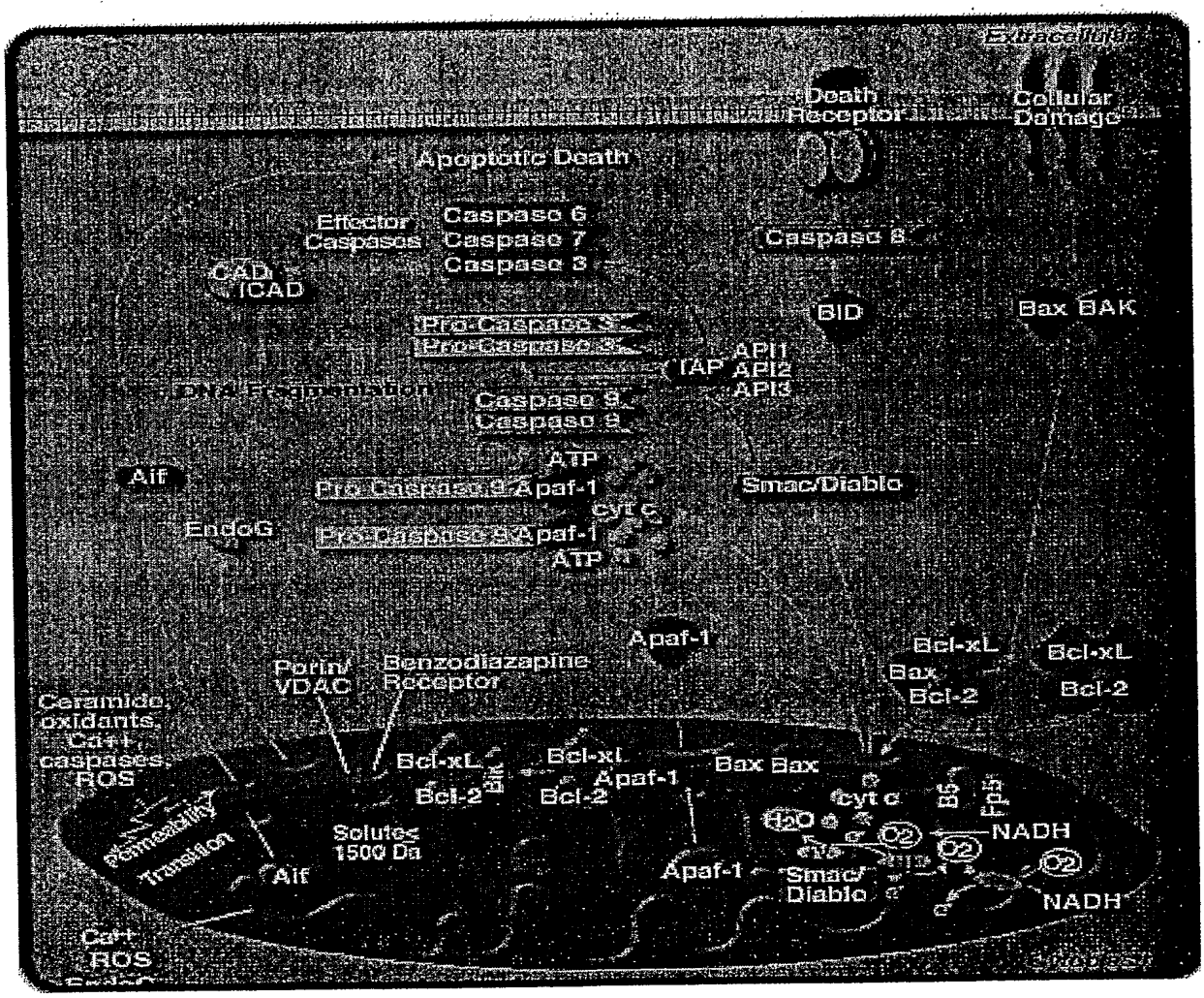
Extracellular
Death Receptor
Cellular Damage
Apoptotic Death
Effector Caspases
Caspase 6
Caspase 7
Caspase 3
Caspase 8
CAD
ICAD
BID
Bax BAK
Pro-Caspase 3
Pro-Caspase 8
IAP
API1
API2
API3
DNA Fragmentation
Caspase 9
Caspase 9
ATP
Aif
Pro Caspase 9 Apaf-1
Smac/Diablo
cyt c
EndoG
Pro Caspase 9 Apaf-1
ATP
Apaf-1
Bcl-xL
Bcl-xL
Porin/ VDAC
Benzodiazapine Receptor
Bax
Bcl-2
Bcl-2
Ceramide, oxidants, Ca++, caspases, ROS
Bcl-xL
Bcl-xl
Bax Bax
Bcl-2
Bcl-2
Apaf-1
cyt c
BS
Fp3
H2O
O2
NADH
permeability Transition
Solutes< 1500 Da
Aif
Apaf-1
Smac/ Diablo
O2
O2
NADH
Ca++ ROS

Figure 1

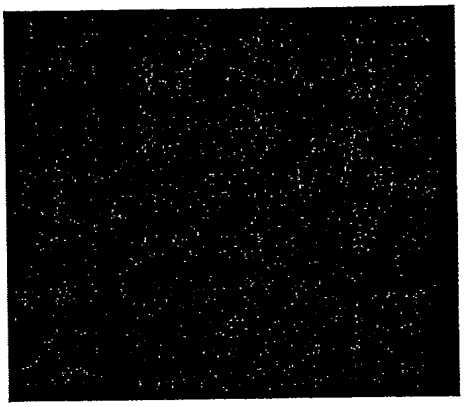

Figure 2 a)

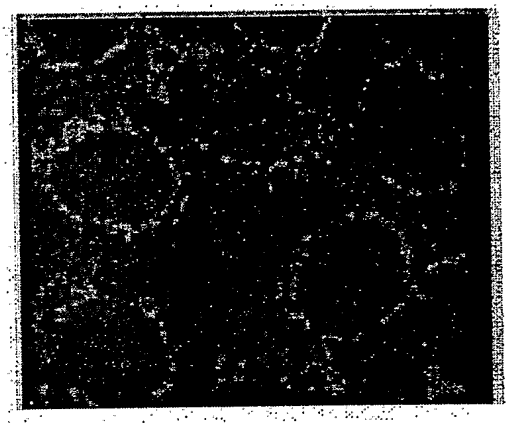

Figure 2 b)

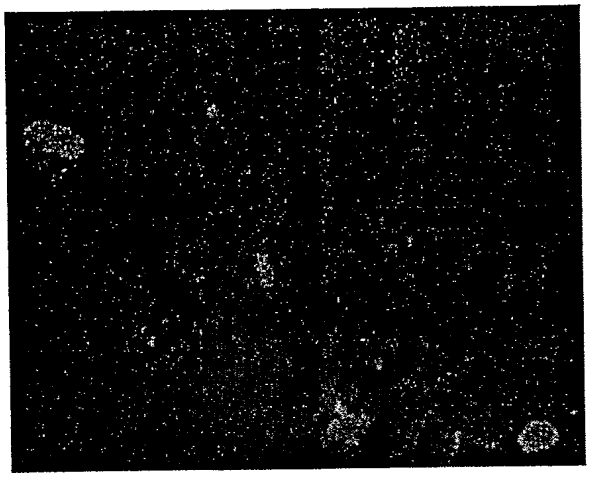

Figure 3 a)

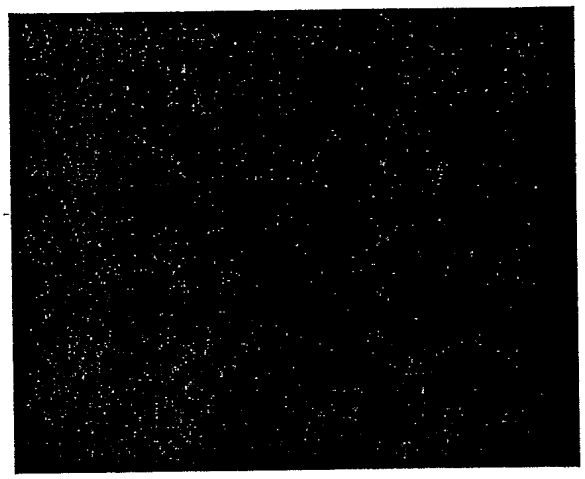

Figure 3 b)

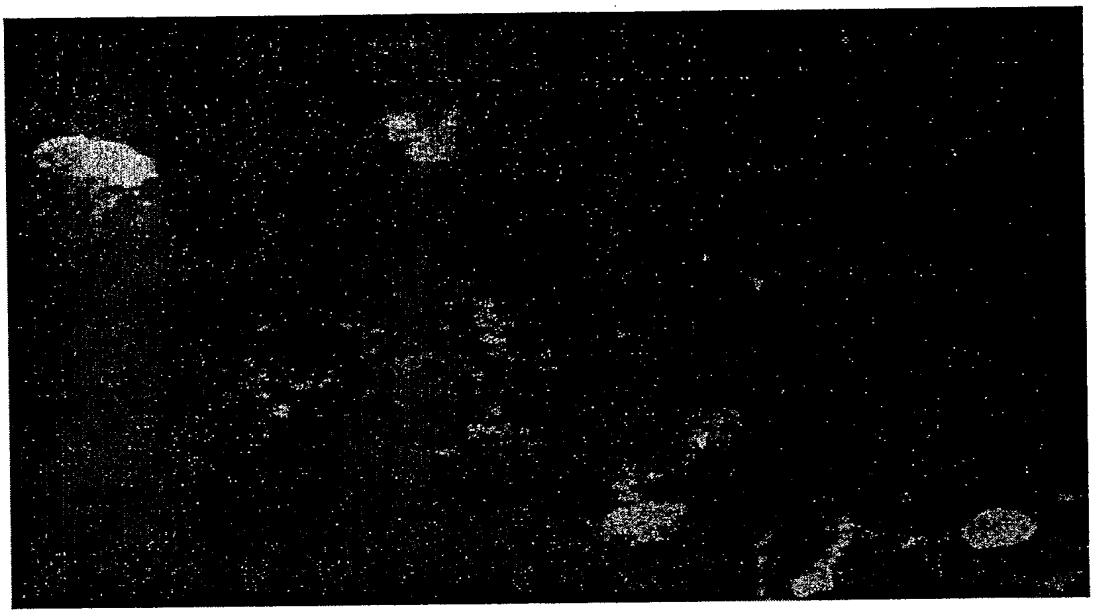

Figure 3c

Figure 4: SEQ ID NO: 1 – Wild Type Cytochrome C (NM_018947)

```
     Met Gly Asp Val Glu Lys Gly Lys Lys Ile Phe Ile Met Lys Cys Ser
 1   ATG GGT GAT GTT GAG AAA GGC AAG AAG ATT TTT ATT ATG AAG TGT TCC

     Gln Cys His Thr Val Glu Lys Gly Gly Lys His Lys Thr Gly Pro Asn
49   CAG TGC CAC ACC GTT GAA AAG GGA GGC AAG CAC AAG ACT GGG CCA AAT

     Leu His Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Pro Gly Tyr Ser
97   CTC CAT GGT CTC TTT GGG CGG AAG ACA GGT CAG GCC CCT GGA TAC TCT

     Tyr Thr Ala Ala Asn Lys Asn Lys Gly Ile Ile Trp Gly Glu Asp Thr
145  TAC ACA GCC GCC AAT AAG AAC AAA GGC ATC ATC TGG GGA GAG GAT ACA

     Leu Met Glu Tyr Leu Glu Asn Pro Lys Lys Tyr Ile Pro Gly Thr Lys
193  CTG ATG GAG TAT TTG GAG AAT CCC AAG AAG TAC ATC CCT GGA ACA AAA

     Met Ile Phe Val Gly Ile Lys Lys Lys Glu Glu Arg Ala Asp Leu Ile
241  ATG ATC TTT GTC GGC ATT AAG AAG AAG GAA GAA AGG GCA GAC TTA ATA

     Ala Tyr Leu Lys Lys Ala Thr Asn Glu
289  GCT TAT CTC AAA AAA GCT ACT AAT GAG
```

Figure 5. SEQ ID NO: 2 – Wild type Cytochrome C (NM_018947)

1
Met Gly Asp Val Glu Lys Gly Lys Lys Ile Phe Ile Met Lys Cys Ser
17
Gln Cys His Thr Val Glu Lys Gly Gly Lys His Lys Thr Gly Pro Asn
33
Leu His Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Pro Gly Tyr Ser
49
Tyr Thr Ala Ala Asn Lys Asn Lys Gly Ile Ile Trp Gly Glu Asp Thr
65
Leu Met Glu Tyr Leu Glu Asn Pro Lys Lys Tyr Ile Pro Gly Thr Lys
81
Met Ile Phe Val Gly Ile Lys Lys Lys Glu Glu Arg Ala Asp Leu Ile
97
Ala Tyr Leu Lys Lys Ala Thr Asn Glu

Figure 6. SEQ ID NO: 3 TriGFP-cytochrome C K72A

```
    Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
1   ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT

    Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
49  GAA TTA GAT GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG

    Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
97  GGT GAA GGT GAT GCA ACA TAC GGA AAA CTT ACC CTT AAA TTT ATT TGC

    Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu
145 ACT ACT GGA AAA CTA CCT GTT CCA TGG CCA ACA CTT GTC ACT ACT CTC

    Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg
193 TCT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA CGG

    His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
241 CAT GAC TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGA

    Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
289 ACT ATA TTT TTC AAA GAT GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC

    Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
337 AAG TTT GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT

    Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
385 GAT TTT AAA GAA GAT GGA AAC ATT CTT GGA CAC AAA TTG GAA TAC AAC

    Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
433 TAT AAC TCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA

    Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Gly Val
481 ATC AAA GTT AAC TTC AAA ATT AGA CAC AAC ATT GAA GAT GGA GGC GTT

    Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
529 CAA CTA GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT

    Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
577 GTC CTT TTA CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG

    Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Gly Phe Val
625 AAA GAT CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GGC TTT GTA

    Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys Leu Glu
673 ACA GCT GCT GGG ATT ACA CAT GGC ATG GAT GAA CTA TAC AAA CTC GAG

    Asn Ser Thr Met Gly Asp Val Glu Lys Gly Lys Lys Ile Phe Ile Met
721 AAT TCG ACC ATG GGT GAT GTT GAG AAA GGC AAG AAG ATT TTT ATT ATG

    Lys Cys Ser Gln Cys His Thr Val Glu Lys Gly Gly Lys His Lys Thr
769 AAG TGT TCC CAG TGC CAC ACC GTT GAA AAG GGA GGC AAG CAC AAG ACT

    Gly Pro Asn Leu His Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Pro
817 GGG CCA AAT CTC CAT GGT CTC TTT GGG CGG AAG ACA GGT CAG GCC CCT

    Gly Tyr Ser Tyr Thr Ala Ala Asn Lys Asn Lys Gly Ile Ile Trp Gly
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```
865   GGA TAC TCT TAC ACA GCC GCC AAT AAG AAC AAA GGC ATC ATC TGG GGA

      Glu Asp Thr Leu Met Glu Tyr Leu Glu Asn Pro Ala Lys Tyr Ile Pro
913   GAG GAT ACA CTG ATG GAG TAT TTG GAG AAT CCC GCC AAG TAC ATC CCT

      Gly Thr Lys Met Ile Phe Val Gly Ile Lys Lys Lys Glu Glu Arg Ala
961   GGA ACA AAA ATG ATC TTT GTC GGC ATT AAG AAG AAG GAA GAA AGG GCA

      Asp Leu Ile Ala Tyr Leu Lys Lys Ala Thr Asn Glu
1009  GAC TTA ATA GCT TAT CTC AAA AAA GCT ACT AAT GAG
```

Figure 7: SEQ ID NO: 4 - triGFP-cytochrome C K72A

1
Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
17
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
33
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
49
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu
65
Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg
81
His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
97
Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
113
Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
129
Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
145
Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
161
Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Gly Val
177
Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
193
Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
209
Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Gly Phe Val
225
Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys Leu Glu
241
Asn Ser Thr Met Gly Asp Val Glu Lys Gly Lys Lys Ile Phe Ile Met
257
Lys Cys Ser Gln Cys His Thr Val Glu Lys Gly Gly Lys His Lys Thr
273
Gly Pro Asn Leu His Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Pro
289
Gly Tyr Ser Tyr Thr Ala Ala Asn Lys Asn Lys Gly Ile Ile Trp Gly
305
Glu Asp Thr Leu Met Glu Tyr Leu Glu Asn Pro Ala Lys Tyr Ile Pro
321
Gly Thr Lys Met Ile Phe Val Gly Ile Lys Lys Lys Glu Glu Arg Ala
337
Asp Leu Ile Ala Tyr Leu Lys Lys Ala Thr Asn Glu

Figure 8. SEQ ID NO: 5 - Cytochrome C (K72A)-triGFP

```
   Met Gly Asp Val Glu Lys Gly Lys Lys Ile Phe Ile Met Lys Cys Ser
 1 ATG GGT GAT GTT GAG AAA GGC AAG AAG ATT TTT ATT ATG AAG TGT TCC

   Gln Cys His Thr Val Glu Lys Gly Gly Lys His Lys Thr Gly Pro Asn
49 CAG TGC CAC ACC GTT GAA AAG GGA GGC AAG CAC AAG ACT GGG CCA AAT

   Leu His Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Pro Gly Tyr Ser
97 CTC CAT GGT CTC TTT GGG CGG AAG ACA GGT CAG GCC CCT GGA TAC TCT

   Tyr Thr Ala Ala Asn Lys Asn Lys Gly Ile Ile Trp Gly Glu Asp Thr
145 TAC ACA GCC GCC AAT AAG AAC AAA GGC ATC ATC TGG GGA GAG GAT ACA

   Leu Met Glu Tyr Leu Glu Asn Pro Ala Lys Tyr Ile Pro Gly Thr Lys
193 CTG ATG GAG TAT TTG GAG AAT CCC GCC AAG TAC ATC CCT GGA ACA AAA

   Met Ile Phe Val Gly Ile Lys Lys Lys Glu Glu Arg Ala Asp Leu Ile
241 ATG ATC TTT GTC GGC ATT AAG AAG AAG GAA GAA AGG GCA GAC TTA ATA

   Ala Tyr Leu Lys Lys Ala Thr Asn Glu Gly Arg Pro Gly Met Ser Lys
289 GCT TAT CTC AAA AAA GCT ACT AAT GAG GGT CGA CCC GGG ATG AGT AAA

   Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp
337 GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT GAA TTA GAT

   Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly
385 GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG GGT GAA GGT

   Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly
433 GAT GCA ACA TAC GGA AAA CTT ACC CTT AAA TTT ATT TGC ACT ACT GGA

   Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Ser Tyr Gly
481 AAA CTA CCT GTT CCA TGG CCA ACA CTT GTC ACT ACT CTC TCT TAT GGT

   Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg His Asp Phe
529 GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA CGG CAT GAC TTT

   Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe
577 TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGA ACT ATA TTT

   Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu
625 TTC AAA GAT GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC AAG TTT GAA

   Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys
673 GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT GAT TTT AAA

   Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser
721 GAA GAT GGA AAC ATT CTT GGA CAC AAA TTG GAA TAC AAC TAT AAC TCA

   His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val
769 CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA ATC AAA GTT

   Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Gly Val Gln Leu Ala
817 AAC TTC AAA ATT AGA CAC AAC ATT GAA GAT GGA GGC GTT CAA CTA GCA

   Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu
```

```
865 GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT GTC CTT TTA

    Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro
913 CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG AAA GAT CCC

    Asn Glu Lys Arg Asp His Met Val Leu Leu Gly Phe Val Thr Ala Ala
961 AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GGC TTT GTA ACA GCT GCT

    Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
1009 GGG ATT ACA CAT GGC ATG GAT GAA CTA TAC AAA
```

Figure 9. SEQ ID NO: 6 - Cytochrome C (K72A)-triGFP

1
Met Gly Asp Val Glu Lys Gly Lys Lys Ile Phe Ile Met Lys Cys Ser
17
Gln Cys His Thr Val Glu Lys Gly Gly Lys His Lys Thr Gly Pro Asn
33
Leu His Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Pro Gly Tyr Ser
49
Tyr Thr Ala Ala Asn Lys Asn Lys Gly Ile Ile Trp Gly Glu Asp Thr
65
Leu Met Glu Tyr Leu Glu Asn Pro Ala Lys Tyr Ile Pro Gly Thr Lys
81
Met Ile Phe Val Gly Ile Lys Lys Lys Glu Glu Arg Ala Asp Leu Ile
97
Ala Tyr Leu Lys Lys Ala Thr Asn Glu Gly Arg Pro Gly Met Ser Lys
113
Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp
129
Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly
145
Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly
161
Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Ser Tyr Gly
177
Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg His Asp Phe
193
Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe
209
Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu
225
Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys
241
Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser
257
His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val
273
Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Gly Val Gln Leu Ala
289
Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu
305
Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro
321
Asn Glu Lys Arg Asp His Met Val Leu Leu Gly Phe Val Thr Ala Ala
337
Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys

Figure 10. SEQ ID NO: 7 - TriGFP- wild type cytochrome C

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      Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
    1 ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT

      Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
   49 GAA TTA GAT GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG

      Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
   97 GGT GAA GGT GAT GCA ACA TAC GGA AAA CTT ACC CTT AAA TTT ATT TGC

      Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu
  145 ACT ACT GGA AAA CTA CCT GTT CCA TGG CCA ACA CTT GTC ACT ACT CTC

      Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg
  193 TCT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA CGG

      His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
  241 CAT GAC TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGA

      Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
  289 ACT ATA TTT TTC AAA GAT GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC

      Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
  337 AAG TTT GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT

      Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
  385 GAT TTT AAA GAA GAT GGA AAC ATT CTT GGA CAC AAA TTG GAA TAC AAC

      Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
  433 TAT AAC TCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA

      Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Gly Val
  481 ATC AAA GTT AAC TTC AAA ATT AGA CAC AAC ATT GAA GAT GGA GGC GTT

      Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
  529 CAA CTA GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT

      Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
  577 GTC CTT TTA CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG

      Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Gly Phe Val
  625 AAA GAT CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GGC TTT GTA

      Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys Leu Glu
  673 ACA GCT GCT GGG ATT ACA CAT GGC ATG GAT GAA CTA TAC AAA CTC GAG

      Asn Ser Thr Met Gly Asp Val Glu Lys Gly Lys Lys Ile Phe Ile Met
  721 AAT TCG ACC ATG GGT GAT GTT GAG AAA GGC AAG AAG ATT TTT ATT ATG

      Lys Cys Ser Gln Cys His Thr Val Glu Lys Gly Gly Lys His Lys Thr
  769 AAG TGT TCC CAG TGC CAC ACC GTT GAA AAG GGA GGC AAG CAC AAG ACT

      Gly Pro Asn Leu His Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Pro
  817 GGG CCA AAT CTC CAT GGT CTC TTT GGG CGG AAG ACA GGT CAG GCC CCT

      Gly Tyr Ser Tyr Thr Ala Ala Asn Lys Asn Lys Gly Ile Ile Trp Gly
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865   GGA TAC TCT TAC ACA GCC GCC AAT AAG AAC AAA GGC ATC ATC TGG GGA

      Glu Asp Thr Leu Met Glu Tyr Leu Glu Asn Pro Lys Lys Tyr Ile Pro
913   GAG GAT ACA CTG ATG GAG TAT TTG GAG AAT CCC AAG AAG TAC ATC CCT

      Gly Thr Lys Met Ile Phe Val Gly Ile Lys Lys Lys Glu Glu Arg Ala
961   GGA ACA AAA ATG ATC TTT GTC GGC ATT AAG AAG AAG GAA GAA AGG GCA

      Asp Leu Ile Ala Tyr Leu Lys Lys Ala Thr Asn Glu
1009  GAC TTA ATA GCT TAT CTC AAA AAA GCT ACT AAT GAG
```

Figure 11. SEQ ID NO: 8 - TriGFP-wild type cytochrome C

1
Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
17
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
33
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
49
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu
65
Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg
81
His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
97
Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
113
Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
129
Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
145
Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
161
Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Gly Val
177
Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
193
Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
209
Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Gly Phe Val
225
Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys Leu Glu
241
Asn Ser Thr Met Gly Asp Val Glu Lys Gly Lys Lys Ile Phe Ile Met
257
Lys Cys Ser Gln Cys His Thr Val Glu Lys Gly Gly Lys His Lys Thr
273
Gly Pro Asn Leu His Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Pro
289
Gly Tyr Ser Tyr Thr Ala Ala Asn Lys Asn Lys Gly Ile Ile Trp Gly
305
Glu Asp Thr Leu Met Glu Tyr Leu Glu Asn Pro Lys Lys Tyr Ile Pro
321
Gly Thr Lys Met Ile Phe Val Gly Ile Lys Lys Lys Glu Glu Arg Ala
337
Asp Leu Ile Ala Tyr Leu Lys Lys Ala Thr Asn Glu

Figure 12. SEQ ID NO: 9 - Wild type Cytochrome C-triGFP

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   Met Gly Asp Val Glu Lys Gly Lys Lys Ile Phe Ile Met Lys Cys Ser
 1 ATG GGT GAT GTT GAG AAA GGC AAG AAG ATT TTT ATT ATG AAG TGT TCC

   Gln Cys His Thr Val Glu Lys Gly Gly Lys His Lys Thr Gly Pro Asn
49 CAG TGC CAC ACC GTT GAA AAG GGA GGC AAG CAC AAG ACT GGG CCA AAT

   Leu His Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Pro Gly Tyr Ser
97 CTC CAT GGT CTC TTT GGG CGG AAG ACA GGT CAG GCC CCT GGA TAC TCT

   Tyr Thr Ala Ala Asn Lys Asn Lys Gly Ile Ile Trp Gly Glu Asp Thr
145 TAC ACA GCC GCC AAT AAG AAC AAA GGC ATC ATC TGG GGA GAG GAT ACA

   Leu Met Glu Tyr Leu Glu Asn Pro Lys Lys Tyr Ile Pro Gly Thr Lys
193 CTG ATG GAG TAT TTG GAG AAT CCC AAG AAG TAC ATC CCT GGA ACA AAA

   Met Ile Phe Val Gly Ile Lys Lys Lys Glu Glu Arg Ala Asp Leu Ile
241 ATG ATC TTT GTC GGC ATT AAG AAG AAG GAA GAA AGG GCA GAC TTA ATA

   Ala Tyr Leu Lys Lys Ala Thr Asn Glu Gly Arg Pro Gly Met Ser Lys
289 GCT TAT CTC AAA AAA GCT ACT AAT GAG GGT CGA CCC GGG ATG AGT AAA

   Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp
337 GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT GAA TTA GAT

   Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly
385 GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG GGT GAA GGT

   Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly
433 GAT GCA ACA TAC GGA AAA CTT ACC CTT AAA TTT ATT TGC ACT ACT GGA

   Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Ser Tyr Gly
481 AAA CTA CCT GTT CCA TGG CCA ACA CTT GTC ACT ACT CTC TCT TAT GGT

   Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg His Asp Phe
529 GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA CGG CAT GAC TTT

   Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe
577 TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGA ACT ATA TTT

   Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu
625 TTC AAA GAT GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC AAG TTT GAA

   Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys
673 GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT GAT TTT AAA

   Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser
721 GAA GAT GGA AAC ATT CTT GGA CAC AAA TTG GAA TAC AAC TAT AAC TCA

   His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val
769 CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA ATC AAA GTT

   Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Gly Val Gln Leu Ala
817 AAC TTC AAA ATT AGA CAC AAC ATT GAA GAT GGA GGC GTT CAA CTA GCA

   Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu
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865 GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT GTC CTT TTA

    Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro
913 CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG AAA GAT CCC

    Asn Glu Lys Arg Asp His Met Val Leu Leu Gly Phe Val Thr Ala Ala
961 AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GGC TTT GTA ACA GCT GCT

    Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
1009 GGG ATT ACA CAT GGC ATG GAT GAA CTA TAC AAA
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Figure 13. SEQ ID NO: 10 - Wild Type Cytochrome C-triGFP

1
Met Gly Asp Val Glu Lys Gly Lys Lys Ile Phe Ile Met Lys Cys Ser
17
Gln Cys His Thr Val Glu Lys Gly Gly Lys His Lys Thr Gly Pro Asn
33
Leu His Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Pro Gly Tyr Ser
49
Tyr Thr Ala Ala Asn Lys Asn Lys Gly Ile Ile Trp Gly Glu Asp Thr
65
Leu Met Glu Tyr Leu Glu Asn Pro Lys Lys Tyr Ile Pro Gly Thr Lys
81
Met Ile Phe Val Gly Ile Lys Lys Lys Glu Glu Arg Ala Asp Leu Ile
97
Ala Tyr Leu Lys Lys Ala Thr Asn Glu Gly Arg Pro Gly Met Ser Lys
113
Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp
129
Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly
145
Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly
161
Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Ser Tyr Gly
177
Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg His Asp Phe
193
Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe
209
Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu
225
Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys
241
Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser
257
His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val
273
Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Gly Val Gln Leu Ala
289
Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu
305
Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro
321
Asn Glu Lys Arg Asp His Met Val Leu Leu Gly Phe Val Thr Ala Ala
337
Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys